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Male-specific asymmetric curvature of anal fin in a viviparous teleost, *Xenotoca eiseni*

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ABSTRACT

Xenotoca eiseni is a viviparous teleost from the family Goodeidae. Internal fertilization occurs in this species; therefore, the male fish must transfer their sperm to the females. To this end, the males use their anal fins as external genitalia during mating. However, in goodeid species, there are a few reports of anal fin modifications functioning as genitalia. In the present study, I describe a male-specific lateral curvature with fin robe thickening on the two most posterior rays of the anal fin of *X. eiseni*. Morphological and behavioral analyses suggested that the lateral curvature provides a directional preference for coupling of *male X. eiseni*. The modification appears to be a male secondary sex characteristic. Other goodeid species *Xenotoca melanosoma* and *Chapalichthys pardalis* also possess the lateral curvature, but without robe thickening. Furthermore, *Zoogoneticus quitzeoensis* exhibited only curvature structure either smaller than those of other species or absent. Therefore, the anal fin curvature may not necessarily be used for internal fertilization in all species investigated in this study. However, it is still possible that it provides novel advantages during mating in certain goodeid species.

1. Introduction

In most viviparous or ovoviviparous species, the males have a mechanism for transporting sperm to the female body. In the early developmental stages of tetrapods, male genitalia develop from genital tubercles and form intromittent organs (Wilhelm and Koopman, 2006). Their morphologies vary among taxa. There is the penis in mammals, crocodilians, and turtles, and the hemipenis in squamates (Thomas et al., 2015). In viviparous cartilaginous fish, the males have a pair of claspers, which develop from the pelvic fins after birth; these structures constitute a secondary sex characteristic (Kajiura et al., 2005).

There are > 500 viviparous teleost species distributed across 14 families (Wourms, 1981). It is presumed that the males inject sperm into the females for internal fertilization. However, it is largely unclear which structures serve as external genitalia. The Cyprinodontiformes include ˜170 viviparous species distributed among the Poeciliidae, Anablepidae, and Goodeidae (Wourms, 1981). Compared with other viviparous teleosts, these species are easily obtained for morphological and/or ecological research due to their small size and abundance in ponds and rivers.

Previous studies have reported distinct modifications of the anal fins as intromittent organs in viviparous species of the Poeciliidae and Anablepidae. Certain viviparous species of the Poeciliidae have an elongated anal fin called a gonopodium (Turner, 1941; Chambers, 1987; see also Fig. 1B). During copulation, the male uses the gonopodium to transport its sperm to the female (Rosa-Molinar et al., 1996). The gonopodium is a secondary sex characteristic formed under the control of androgenic hormones (Turner, 1942; Ogino et al., 2004). In *Anableps anableps* (four-eyed fish; Anablepidae), the anal fin twists laterally into a tube to serve as an intromittent organ (Turner, 1950; see also Fig. 1A). The direction of curvature relative to the midline of the fish may determine compatibility with the female, which has an asymmetric orifice in the urogenital sinus (Bisazza et al., 1998). To the best of my knowledge, however, no experimental investigations of biological significance of the asymmetry have been published. Furthermore, the regulatory mechanisms and heritability of the asymmetric structures in these genital organs are unknown.

The Goodeidae includes 42 viviparous species distributed in the lakes and rivers of North America (Foster and Piller, 2018). Previous studies indicated that these goodeids have short gonopodia (also known as “andropodia”) in a separate anterior part of the anal fin. The anterior lobe includes seven rays, of which the first is rudimentary and rays 2–7 are functional (Turner, 1933; Hubbs and Turner, 1939; Parenti, 1981; see also Fig. 1A). The short lobe of the fin wraps around the female genital orifice during mating (Nelson, 1975; Greven and Brenner, 2010). No other anal fin modifications have been previously reported for male goodeid fish.

In the present study, I explore other anal fin traits in male goodeids,

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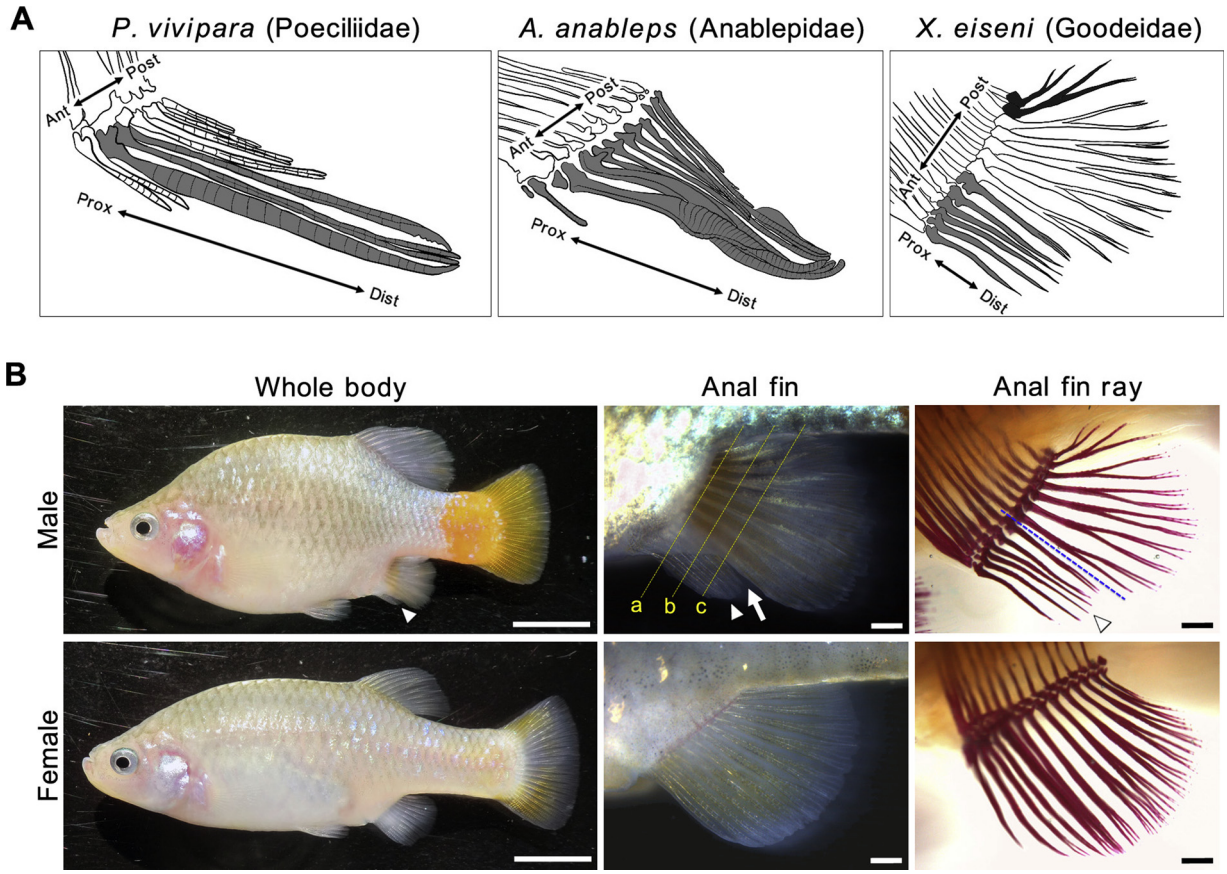

Fig. 1. Anal fin structures of viviparous species belonging to the order Cyprinodontiformes.
A. Anal fins of *Poecilia vivipara* (after Parenti, 1981), *Anableps anableps* (after Turner, 1950), and *X. eiseni*. Gray shading indicates the formation of gonopodia from anal fin rays in each species. Black shading shows lateral-turning anal fin rays in *X. eiseni* (see also Fig. 2C). Ant, anterior. Post, posterior. Prox, proximal. Dist, distal. **B.** Morphological comparisons of adult male and female *X. eiseni*. Whole-body and anal fin photographs were taken on anesthetized samples. Anal fin rays were stained with alizarin red. Arrowheads indicate the short gonopodia including the six most anterior rays. Arrows and blue dotted lines indicate the border between the gonopodium and the rest of the fin (notch). Yellow dotted lines and lowercase letters indicate the approximate loci of the sections corresponding to Fig. 2C. Scale bars: 10 mm (whole body); 1 mm (the other parts).

specifically, *Xenotoca eiseni* (redtail splitfin; Rutter, 1896), and report a lateral curvature on the posterior side of the anal fin. I describe the structure and development of this anal fin modification in *X. eiseni* and compare its fin shapes with those of related goodeid species, *Xenotoca melanosoma* (black splitfin; Fitzsimons, 1972), *Zoogoneticus quitzeoensis* (picotee splitfin; Bean, 1898), and *Chapalichthys pardalis* (polka-dot splitfin; Álvarez del Villar, 1963). The four species are reported as related species according to a molecular analysis (Webb et al., 2004).

2. Materials and Methods

2.1. Animal experiments

The study design was approved by the ethics review board of the Center for Animal Experiments of the Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto, Japan (Approval No. J-9-2). A minimal number of experimental animals was sacrificed using 0.01% tricaine as the anesthetic. All methods applied in this study were performed according to relevant guidelines and regulations.

2.2. Fish breeding

X. eiseni, *X. melanosoma*, *Z. quitzeoensis*, and *C. pardalis* were purchased from Meito Suien Co. Ltd. (Nagoya, Japan). Adult fish were maintained in freshwater at 27 °C under a 14 h/10 h light/dark photoperiod. Sample fish had been maintained by group breeding over eight generations, without distinction of anal fin morphology. Juveniles were fed live brine shrimp larvae and Hikari Rabo 450 Fish Food. Adults were fed Hikari Crest Micro Pellets (Kyorin Co. Ltd., Himeji, Japan). Unless otherwise stated, I used mature fertile fish (3–5 months old) for all morphological and histological observations.

2.3. Histology

The fish samples were fixed for one week with 3.7% formalin at room temperature (approximately 25 °C). Paraffin sections and hematoxylin-eosin (HE) staining were performed by Biopathology Institute Co. Ltd. (Kunisaki, Japan).

2.4. Microscopy

Live fish samples were anesthetized with 0.01% tricaine on ice for 30–60 min. Microscopic observations were made with a Leica M205C microscope fitted with a Leica DFC300 FX digital color camera (Leica Microsystems, Mannheim, Germany). Histological samples were photographed with an Olympus BX50 microscope fitted with UPLAPO 4X (numerical aperture 0.16) and 10X (numerical aperture 0.4) objective lenses and an Olympus DP72 Microscope Digital Camera (Olympus Corp., Tokyo, Japan).

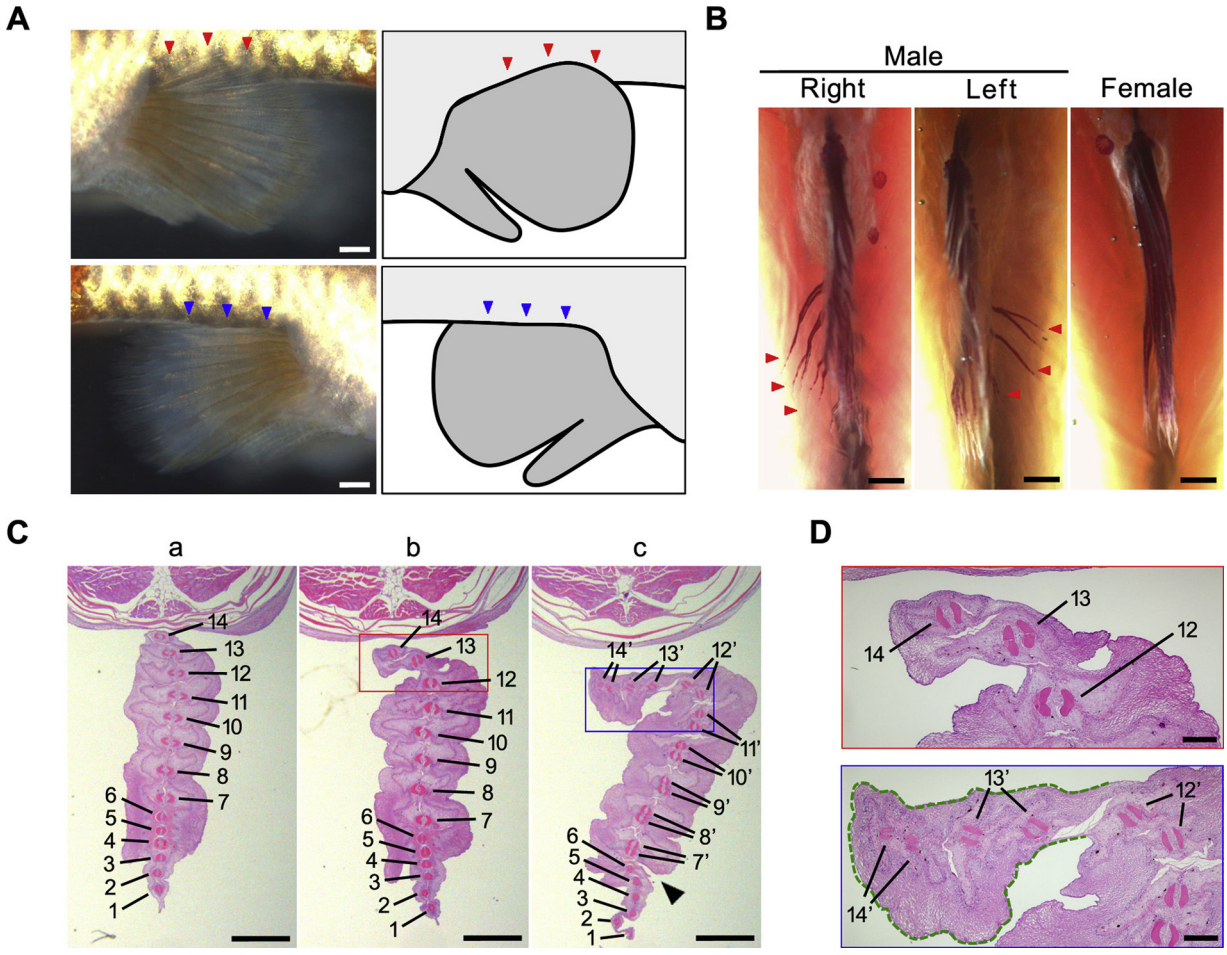

Fig. 2. Curvature modification in anal fin of *Xenotoca eiseni*.
A. Photographs and traced images of male anal fin indicating its curvature. Upper panels show left lateral views indicating that the anal fin has turned towards the near side (red inverted triangles). Lower panels show right lateral view in which the anal fin has turned towards the far side (blue inverted triangles). Scale bars: 1 mm. **B.** Ventral view of male and female anal fins stained with alizarin red. Red triangles indicate curved fin rays. Male fish are classified according to right- or left-oriented anal fin curvature. Curvature is absent in the anal fin rays of female fish. Scale bars: 1 mm. **C.** HE-stained transverse sections of male anal fins. Lowercase letters indicate sectional loci corresponding to Fig. 1A. Numbers indicate fin rays, and numbers with an apostrophe indicate branched fin rays at their distal tips. The six most anterior rays form a short separate gonopodium. Black arrowhead indicates a notch between the sixth and seventh rays. Scale bars: 1 mm. **D.** Enlarged images of the posterior end of the anal fin are indicated by red and blue squares in Fig. 2C. Green dotted line indicates node-like structure. Scale bars: 200 μm.

2.5. Behavioral analysis

Two mature *X. eiseni* individuals were released into a mating tank separated by an acrylic board one day before recording. The next morning, immediately after turning on the room light, the separator was removed and their behavior was recorded using the HDR-CX560 V digital HD video camera recorder (SONY, Tokyo, Japan). Their behavior was recorded for 15–60 min in each experiment. After recording, approach and coupling were visually evaluated by reference to Greven and Brenner, 2010. The recording was performed using different fish in each trial. If coupling was successful, the pair of fish was not used in the later trials. Nine male ([right]:[left] = 4:5) and twelve female littermate fish were used in the behavioral analysis.

3. Results

3.1. Male-specific anal fin modification in X. eiseni

In viviparous species belonging to the order Cyprinodontiformes, noticeable modifications in male anal fin are known to assist in coupling and internal fertilization. Elongated anterior fin rays are visible in viviparous Poeciliidae species. Contrarily, whole-fin curvatures and tubular modifications were observed in the member of Anablepidae. However, these modifications were not observed in our male *X. eiseni* (Fig. 1A). In Goodeidae species, the anterior part of the anal fin, including the six most anterior rays, are separated from the rest of the fin by a notch between the sixth and seventh rays (Fig. 1A and B). This short gonopodia assists their coupling. Certain Goodeidae species such as *X. eiseni* are sexually dimorphic. In *X. eiseni*, the male and female fish are visually indistinguishable at the fry stage. At approximately one month after birth, the male fish exhibit sex-specific traits such as a high-arching body and red tail (Fig. 1B). In this study, I counted only the anal fin rays that absorbed alizarin or hematoxylin-eosin (HE) stain. Therefore, the undeveloped first ray (Turner, 1933; Hubbs and Turner, 1939; Parenti, 1981) was excluded. Based on these criteria, our male *X. eiseni* breeding population had 13–14 rays in their anal fins (Fig. 1B). According to my observation, a slight curvature of the fin rays relative to the midline was found on the posterior side of the anal fin. Therefore, the distal tips of the fin overlapped the trunk (Fig. 2A). These modified fin rays pointed either toward the left or right of the midline (Fig. 2B). On the contrary, there was no anal fin curvature in the female fish (Fig. 2B). In the male anal fin, the 13th and 14th rays curved laterally. The angle increased toward the distal fin ray tips (Fig. 2C; Supplementary Fig. S1). The male anal fin ray membrane was thickened around the fin rays. The 7th to 14th fin rays branched at the distal tips (Fig. 2C). In particular, the distal portion of the fin including the

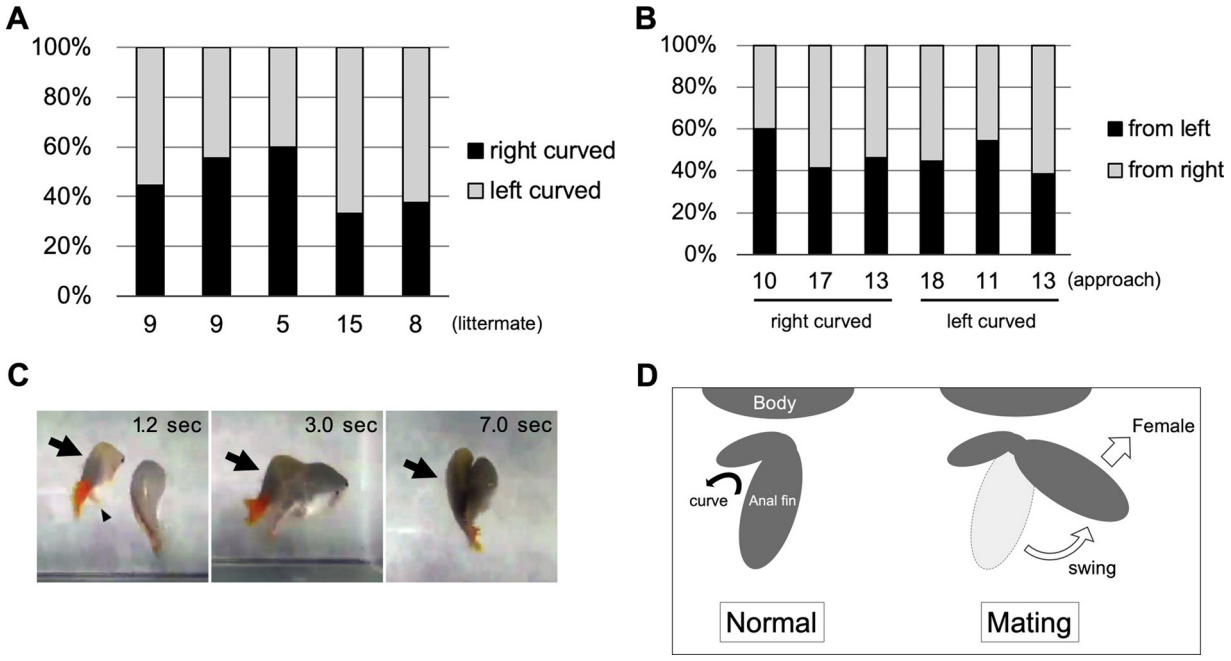

Fig. 3. Characterization of male fish with the curved anal fin.
A. Graph indicates the ratio for left–right direction of the anal fin curvature of littermate male fish from five independent breeding. The parent fish used in these mating were not overlapped. The numerical data are presented in Table 1. **B.** Graph indicates the ratio of approach direction of male fish during mating. The approach direction was counted from the start of mating until the first coupling. Six independent pairs were used in the behavioral record. **C.** Images captured during mating including coupling from Movie 4. A male with a left-curved anal fin swung its fin lobe toward right (1.2 sec) and approached a female fish on the right (3.0 sec). The male fish with left curvature succeeded to couple on the right side of the female (7.0 sec). Arrow indicates male fish, the other is female. Arrowhead indicates the swinging anal fin. **D.** My hypothetical model for an asymmetric motility of anal fin based on the curved modification. The illustration indicates transverse section of the male anal fin of *X. eiseni*. The left-curved anal fin prefers or restricts "right swing" for coupling with a female on the right and vice versa.

Table 1
Ratio of curvature direction in the anal fin of *X. eiseni* male littermates.

Breeding #	Male littermates	Anal fin curvature		Age at observation (month)
		Right	Left	
1	9	4	5	7
2	9	5	4	7
3	5	3	2	6
4	15	5	10	4
5	8	3	5	2.5
Total	46	20	26	

Table displays the numbers of right and left curvatures of the anal fins in littermate male fish from five independent breeding events. This is a numerical source of the graph in Fig. 3A.

branches emanating from the 13th and 14th rays is bent at a sharp angle and appear like a "node" by a noticeable constriction between the 12th and 13th rays (Fig. 2D).

3.2. Roles of anal fin curvature during mating

Based on the anal fin morphology, *X. eiseni* male can be categorized into two subpopulations according to the direction of curvature. To know the appearance ratio of the phenotype in the present breeding population, I counted the male fish in mature male littermates with respect to each curvature direction. No considerable bias was observed in five independent breeding (Fig. 3A and Table 1). The overall left–right ratio was statistically even ([right]:[left] = 20:26; $p = 0.53$, Chi-squared test). To investigate the roles of anal fin modification, I observed mating behavior of fish with left or right curvature. Prior to coupling, the male fish appealed to female fish from sideways (Fig. 3B and Movie S1). There was no significant bias in left–right preference depending on the curvature direction of the anal fin (right curved, [from right]:[from left] = 21:19; left curved, [from right]:[from left] = 23:19; $p = 0.83$, Chi-squared test). The overall left–right ratio for appeal direction was statistically even ([from right]:[from left] = 44:38; $p = 0.63$, Chi-squared test). However, the male–female position during copulation was restricted by the curvature direction of the anal fin in male. The male fish with left-curved fin coupled with female fish only from the right side (Fig. 3C and Movie S2; n = 3) and vice versa (Movie S3; n = 4). Furthermore, I also observed that a male fish with left-curved fin swung the anal fin towards right (Fig. 3C and D; Movie S4). The opposite approach could not succeed in sexual coupling in my observation.

3.3. Developmental onset of anal fin curvature

To identify the stage at which the anal fin curvature modification appeared, anal fin morphologies were compared between the male and female fish, starting from the juvenile stage until the secondary sex characteristics became clearly visible. I obtained *X. eiseni* immediately after birth and observed the shapes of their anal fins thereafter. Up until the 2 weeks after birth, the sex of the fish could not be determined on the basis of their external morphologies. Shape of the anal fin was particularly indistinguishable between the sexes up until 14 d after birth. Approximately 21 d after birth, separation of the anterior fin rays became clearly visible in the male fish (Fig. 4A). By 28 d after birth, testicular differentiation and spermatogenesis had already started (Supplementary Fig. S2). Body shape differentiation and male-specific red tail color became visible from a month after birth. Curvature modification was also observed at the posterior lobe of the anal fin (Fig. 4B; Supplementary Fig. S3). Turning and branching of the two most posterior rays started by 28 d after birth. Nevertheless, fin ray membrane thickening including a node-like posterior lobe emanating from the 13th and 14th rays were not yet observed at this time (Fig. 4C; cf. Supplementary Fig. S6).

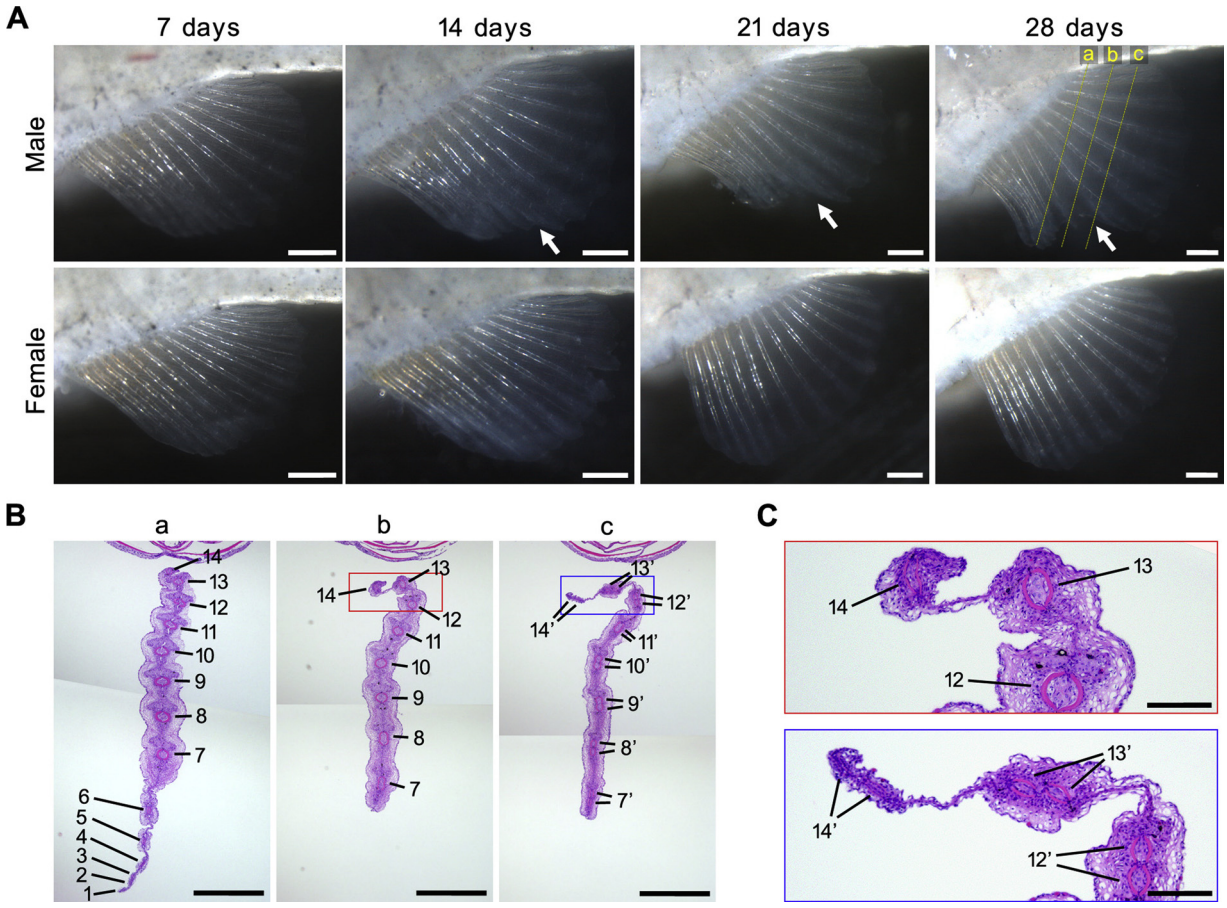

Fig. 4. Morphological changes of the anal fins in the juvenile stages.
A. Time-course photographs showing male and female *X. eiseni* anal fin morphology 7 d, 14 d, 21 d, and 28 d after birth, respectively. Arrows indicate the regions of the notch between the sixth and seventh rays. Yellow dotted lines and lowercase letters indicate approximate sectioning loci corresponding to Fig. 4B. Twelve littermate fish (five males, seven females) were sampled and observed immediately after birth. Scale bars: 500 μm. **B.** Typical image of HE-stained transverse sections of the anal fins of a 28-d male. Lowercase letters indicate sectional loci corresponding to Fig. 4A. The six most anterior rays indicate an immature separate gonopodium. Numbers indicate fin rays, and numbers with an apostrophe indicate branched fin rays at their distal tips. Scale bars: 500 μm. **C.** Enlarged images of the posterior curvature of the fins are indicated by red and blue squares in Fig. 4B. Scale bars: 100 μm.

3.4. Anal fin morphology in other goodeids

A distinct curvature modification was seen in the anal fin of *X. eiseni*. To validate whether this trait is common among goodeids, I also observed and compared the anal fin morphology of *X. melanosoma*, *Z. quitzeoensis*, and *C. pardalis*. I examined both living fertile males and tissues excised from them. Like *X. eiseni*, all of them presented male-specific short gonopodia on the anterior side of their anal fins (Fig. 5A; Supplementary Fig. S4). Histological comparisons of their fin shapes revealed distal branching (Fig. 5B; Supplementary Fig. S5). Distinct curvatures of the posterior parts of the anal fins were also seen in *X. melanosoma* and *C. pardalis*. These species also had two fin rays turned laterally at the posterior edge and their angles of curvature increased distally. However, unlike *X. eiseni*, their anal fins turned at an obtuse angle and lacked thickening not only in the posterior portion but also the whole fin (Fig. 5B; Supplementary Fig. S6). In *Z. quitzeoensis*, the anal fin curvature was either smaller than that in the other species (Fig. 5B).

4. Discussion

In the present study, I describe the detailed structure of the anal fins of the male goodeid *X. eiseni* and those of three related species. Previous studies indicated that the anal fin aids internal fertilization during mating in goodeid species. However, their anterior gonopodia are too short to be effective as intromittent organs unlike those of the members of Poeciliidae and Anablepidae (Nelson, 1975; Greven and Brenner, 2010). Here I describe the possible functions of the novel curvature and node-like structure in the anal fin of *X. eiseni*.

Asymmetric modification of the anal fin in male has also been reported in four-eyed fish belonging to Anablepidae. Their anal fins twist right or left to form tubular gonopodia. The curvature direction creates right–left asymmetries in sexual behavior (Turner, 1950; Burns, 1991). The genital orifice of the female four-eyed fish is an outgrowth from the right or left (Garman, 1895). This asymmetry determines the directional preference during mating. Dextral males must mate with sinistral females and vice versa (Wourms, 1981; Bisazza et al., 1998). In *X. eiseni*, the posterior part of the anal fin turns laterally and forms a node-like structure. Consequently, I hypothesized that the anal fin has a preference direction to swing during mating. The behavioral observation during mating suggested that this restricted lateral bend in the anal fin creates mating and copulatory dextrality or sinistrality similar to that of the four-eyed fish. According to this scenario, male *X. eiseni* acquired the morphological asymmetry in the anal fin to effectively carry their sperm into the female genitalia. The dextrality or sinistrality of the asymmetric structure might be more advantageous for male *X. eiseni* than symmetric ambidextrous. This might be similar to the selective pressures facing of Anablepidae members. However, I am not excluding the possibility that male fish could couple from the conflict side, because they appealed and tried mating from both sides of the

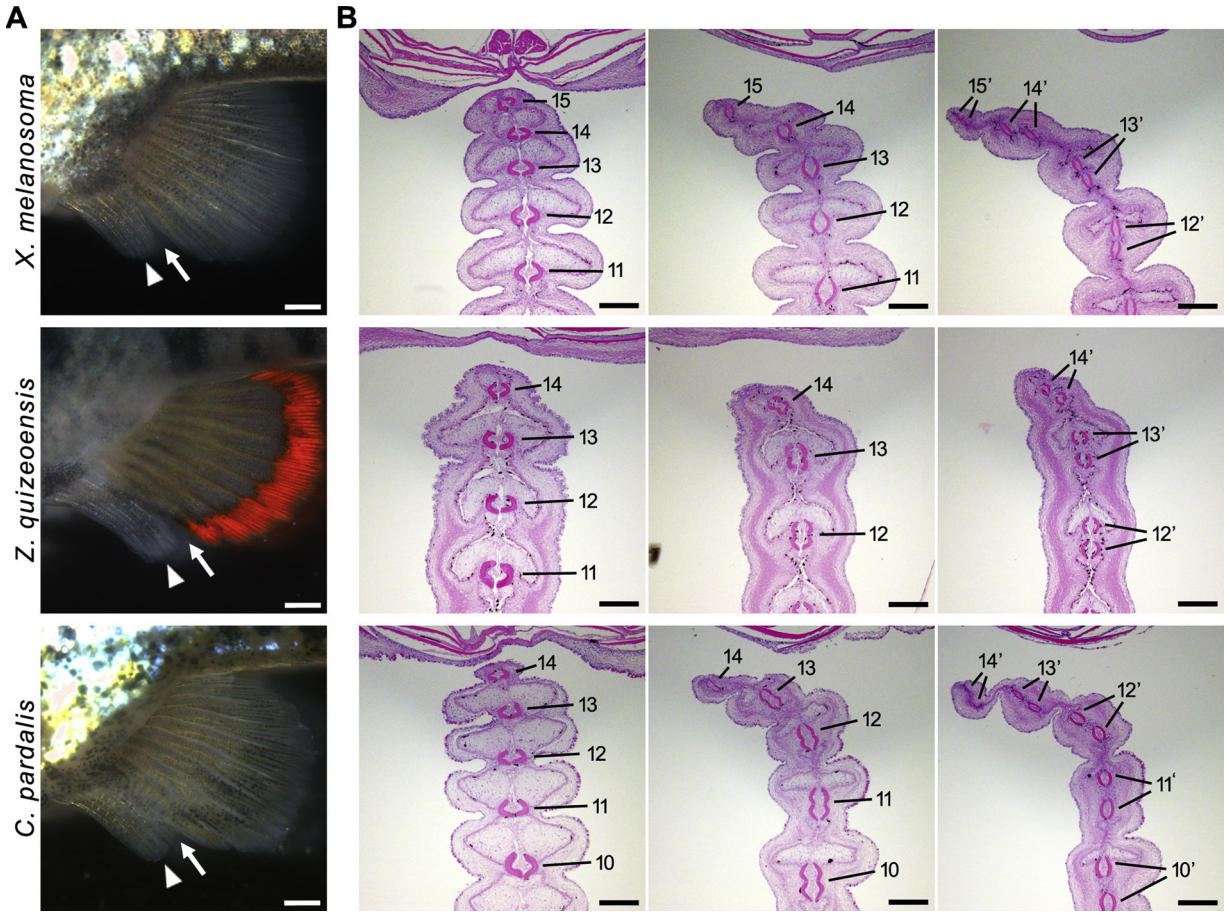

Fig. 5. Morphologies of the anal fins of other goodeid species.
A. Morphological comparison of adult male anal fins of *X. melanosoma*, *Z. quitzeoensis*, and *C. pardalis*. Arrowheads indicate the short gonopodium including the six most anterior rays. Arrows indicate a notch between the gonopodium and the rest of the anal fin. Three males were observed per species. Scale bars: 1 mm. **B.** Typical images of HE-stained transverse sections of the dorsal position of the male anal fins. Numbers indicate fin rays, and numbers with an apostrophe indicate branched fin rays at their distal tips. The three sequential images indicate the sectional positions left: basal, central: medium, and right: distal. Scale bars: 200 μm.

female. Contrarily, I found no asymmetrical positioning of the genital orifice or curvature in the anal fin of female *X. eiseni*, which is similar to the findings of a previous study (Greven and Brenner, 2010). The evidence suggests that asymmetrical modifications provide sexual advantages for only male *X. eiseni*. Another question is whether a fin curved toward to a particular direction is more dominant for natural selection than the other type. In case of left–right preference with respect to predation, asymmetric structure and/or behavior are biased or fluctuated under natural conditions (Hori, 1993; Hoso et al., 2007). In this case, prey–predator interaction acts as a selection pressure. In case of *X. eiseni*, both the dextral and sinistral males could be raised to sexual maturation in my facility. The left–right ratio of the anal fin in males was statistically even in the breeding population. Besides, the female fish could accept courting from both types of male. Therefore, under breeding condition, I presume that there is no selection or competition according to the curvature direction of anal fin. If there is no left- or right-specific selection pressure in natural conditions, both the populations might coexist without competition. To verify the hypothesis, determination of hereditary pattern and/or long-term field research is required.

In fish, gonad development or sex hormonal secretion is regarded as a primary sex characteristic. In contrast, the development of sex-specific organs, such as intromittent organs or ovipositors, is classified as a secondary sex characteristic (Purdom, 1993). The anal fin modification in *X. eiseni* is male-specific, and sexual dimorphism becomes apparent at around one month after birth. A previous study indicated that an androgen-dependent metabolic pathway in the anal fins of juvenile *Gambusia affinis* (Poeciliidae) transforms these structures into elongated gonopodia (Ogino et al., 2004). In the four-eyed fish (*Anableps dowei*), a male-specific tubular gonopodium develops after birth from a fin structure common to both sexes (Burns, 1991). For *X. eiseni*, the lateral turn in the posterior part of the anal fin, the anterior notch, and the short gonopodium form simultaneously. These observations suggest that the male-specific anal fin modification in *X. eiseni* could be regulated as a secondary sex characteristic.

To validate whether the anal fin curvature modification is specific to *X. eiseni*, I also analyzed the goodeids *X. melanosoma*, *Z. quitzeoensis*, and *C. pardalis*. *X. melanosoma* and *C. pardalis* have curvature modifications in the posterior parts of their anal fin but the angles are smaller than that in *X. eiseni*. The anal fin curvature modification of *Z. quitzeoensis* is either smaller than those of the other species. Therefore, asymmetric anal fin curvature may not be essential for mating or internal fertilization in members of Goodeidae including *Z. quitzeoensis*. Besides, *X. melanosoma* and *C. pardalis* exhibit an intermediate phenotype with an obtuse-curved flat fin. I have two hypotheses pertaining to the anal fins of these species. The first is that these species decreased or lost asymmetry as an ancient trait unnecessary for their survival and reproduction. The second is that the current traits are optimized for the reproduction of each species and have not undergone any anal fin modification. To validate the hypotheses, further phylogenetic and morphological analyses using these and other goodeids are required.

A recent study reclassified *X. eiseni* as three species, namely, *X. eiseni*, *Xenotoca doadrioi* (San Marcos redtail splitfin), and *Xenotoca lyonsi* (Tamazula redtail splitfin) according to meristic and morphometric

analyses of their local populations (Domínguez-Domínguez et al., 2016). In this study, I evaluated two independent sources of *X. eiseni* obtained from a breeder in Germany and the Higashiyama Zoo and Botanical Gardens in Japan via a commercial supplier. Both of *X. eiseni* presented with curved anal fins. However, I did not rule out the possibilities that the curved anal fins observed in these populations were dissimilar to those of wild type *X. eiseni* and related species and this trait is unique to the artificially raised populations used in the present study. To investigate the genetic and/or evolutionary origins of the curved anal fin, it is preferable to use wild populations of *X. eiseni* or other species whose origins and genetic backgrounds are well defined.

I also propose the hypothesis that anal fin asymmetry is, in fact, a by-product of anal fin outgrowth. Natural selection may have favored this transformation in viviparous fish. An enlarged anal fin may more effectively transfer sperm to the female genital orifice than one of normal size. Since the space on the posterior side of the anal fin is limited, its terminal portion must be displaced to either side of the caudal peduncle. The results of this study do not preclude the possibility that this anal fin asymmetry is simply a passive deformation that developed as a by-product of an important trait acquired through natural selection.

5. Conclusion

In the present study, I identified a lateral curvature in the anal fin of the males of the goodeid species *X. eiseni*. This male-specific modification becomes apparent at around one month after birth. This is the first report of a curvature modification in the posterior part of the anal fin of *X. eiseni*. However, this anal fin curvature was smaller than those of *X. melanosoma*, *C. pardalis* and *Z. quitzeoensis*. Therefore, this modification may not be a common to all members of the Goodeidae family.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.zool.2019.03.002.

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